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Multilocus tests of Pleistocene refugia and ancient divergence in a pair of Atlantic Forest antbirds (*Myrmeciza*)

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Abstract

The Atlantic Forest (AF) harbours one of the most diverse vertebrate faunas of the world, including 199 endemic species of birds. Understanding the evolutionary processes behind such diversity has become the focus of many recent, primarily single locus, phylogeographic studies. These studies suggest that isolation in forest refugia may have been a major mechanism promoting diversification, although there is also support for a role of riverine and geotectonic barriers, two sets of hypotheses that can best be tested with multilocus data. Here we combined multilocus data (one mtDNA marker and eight anonymous nuclear loci) from two species of parapatric antbirds, *Myrmeciza loricata* and *M. squamosa*, and Approximate Bayesian Computation to determine whether isolation in refugia explains current patterns of genetic variation and their status as independent evolutionary units. Patterns of population structure, differences in intraspecific levels of divergence and coalescent estimates of historical demography fit the predictions of a recently proposed model of refuge isolation in which climatic stability in the northern AF sustains higher diversity and demographic stability than in the southern AF. However, a pre-Pleistocene divergence associated with their abutting range limits in a region of past tectonic activity also suggests a role for rivers or geotectonic barriers. Little or no gene flow between these species suggests the development of reproductive barriers or competitive exclusion. Our results suggests that limited marker sampling in recent AF studies may compromise estimates of divergence times and historical demography, and we discuss the effects of such sampling on this and other studies.

Keywords: anonymous loci, Atlantic Forest, multilocus phylogeography, refugia theory

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Introduction

Neotropical forests are well known for their astonishing diversity, and defining which evolutionary processes originated such diversity has been the goal of evolutionary biologists for more than a century. Several early naturalists and modern biologists have sought to identify patterns and processes, such as the presence of rivers, cycles of climate variation or geologic events, that may have contributed to the build-up of Neotropical diversity (e.g. Wallace 1852; Haffer 1969, 1993, 1997; Bush 1994; Moritz *et al.* 2000; Bates *et al.* 2008; Rull 2008; Carnaval *et al.* 2009; Hoorn *et al.* 2010; Thomé *et al.* 2010). However, complexity seems to be the rule in the Neotropics, and it has become clear that a single model alone or a specific geological period cannot

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explain the origin of the entire Neotropical biota (Bush 1994; Bates *et al.* 2008; Rull 2008; Thomé *et al.* 2010; D'Horta *et al.* 2011; Brumfield 2012).

One of the most diverse—but least studied—areas of the Neotropics is the Atlantic Forest (henceforth referred to as AF). Originally distributed mostly throughout eastern Brazil, Paraguay and Argentina (Rizzini 1997; Silva *et al.* 2004), the AF harbours between two and three per cent of all known vertebrate species and stands as one of the five most diverse areas in the world (Myers *et al.* 2000). Currently reduced to 11.4–16% of its preclearing distribution (Ribeiro *et al.* 2009), the AF historically (i.e. 500 years ago) stretched across 25° of latitude and from sea level to 1700 m of altitude and was isolated from other forest biomes in South America by open vegetation (Silva *et al.* 2004). The evolutionary processes involved in the generation of such diversity, however, are still poorly known. Avian phylogenies suggest a complex evolutionary history of species endemic to the AF. Both local diversification (e.g. *Tangara* tanagers, Burns & Naoki 2004) and diversification involving faunal connections with other Neotropical areas have been identified (e.g. Amazon forest, *Xiphorhynchus* woodcreepers, Cabanne *et al.* 2008; Andes, *Amazon* parrots, Russello & Amato 2004; South American savannahs, buteonine hawks, Amaral *et al.* 2009). Even a single avian radiation may present contrasting histories of AF endemics in space and time (ovenbirds, Furnariidae, Derryberry *et al.* 2011). At the micro-evolutionary level, avian phylogeography in the AF has just began to be explored (e.g. Cabanne *et al.* 2007, 2008, 2011; Mata *et al.* 2009; D'Horta *et al.* 2011; Batalha-Filho *et al.* 2012; Maldonado-Coelho 2012). The high diversity (199) of endemic avian species (Stotz *et al.* 1996) makes birds an excellent group to explore the recent history of the AF.

Refuge isolation has been recently proposed as one of the most powerful forces shaping intraspecific diversity in the AF (Carnaval *et al.* 2009; Carnaval & Moritz 2008; henceforth referred to as the stability-extinction model). Classical refuge theory hypothesizes that forest organisms are isolated in forest patches during periods of pronounced climatic changes that affected life on Earth since the onset of the Quaternary (Haffer 1969; Vanzolini & Williams 1970) and perhaps even earlier (Haffer 1993; Hewitt 1999). Isolation in forest patches surrounded by unsuitable habitat would lead to differentiation, and in some cases, speciation. The stability-extinction model uses present and past models of the distribution of the AF to suggest that current patterns of intraspecific genetic variation are the result of periodic isolation in areas of forest stability (i.e. refuges) and extinction in areas outside refuges during glacial periods, in particular the Last Glacial Maximum (LGM) around 21 000 years ago. Two main refuges are predicted to have existed in the AF during the LGM (Fig. 1); one between the rivers Doce and São Francisco (i.e. the Bahia refuge) and a second one north of the São Francisco River (the Pernambuco refuge). Areas south of the Doce River would have been environmentally less stable during glaciations. Thus, organisms widespread in the AF should have higher genetic diversity, more population structure and increasingly stable historical demographies as one moves north of the Doce River, while populations south of that river should present lower levels of diversity, less population structure and traces of recent recolonization events from northern populations (Carnaval & Moritz 2008; Carnaval *et al.* 2009).

In addition to refuges, it has been suggested that the development of rivers (Pellegrino *et al.* 2005) and other physiographic barriers (Silva & Straube 1996; Brunes *et al.* 2010; Thomé *et al.* 2010) have been important factors shaping diversity in the AF. Those barrier hypotheses differ in expectations from the refuge hypothesis both spatially (breaks should coincide with barriers, not refuges) and temporally (splits could be much older than those caused by refuges). More importantly, in contrast to the expectations under the refuge hypothesis (Moritz *et al.* 2000), major demographic fluctuations should not occur under the barrier hypotheses, because cycles of extinction and expansion are not expected.

The foundations of AF phylogeography were laid by recent studies on AF endemics from several taxonomic groups. Those studies were mostly based on mitochondrial DNA alone or up to two unlinked markers (e.g. Pellegrino *et al.* 2005; Grazziotin *et al.* 2006; Cabanne *et al.* 2007, 2008; Carnaval *et al.* 2009; Fitzpatrick *et al.* 2009; D'Horta *et al.* 2011; Batalha-Filho *et al.* 2012; Maldonado-Coelho 2012), with a few exceptions using three or four independent loci (Tchaicka *et al.* 2007; Martins *et al.* 2009; Brunes *et al.* 2010; Thomé *et al.* 2010; Amaro *et al.* 2012). Theory predicts that inferences based on one or few independent markers may be subject to several locus-specific shortcomings, especially locus-specific stochastic error (see Discussion), which in turn may influence estimates of demographic history, divergence times and population structure (Edwards & Beerli 2000; Hickerson *et al.* 2005, 2006, 2010; Carling & Brumfield 2007; Heled & Drummond 2008; Brito & Edwards 2009). The extent to which those issues affect our current knowledge of AF phylogeography, however, is unknown.

Study system

The Squamate Antbird (*Myrmeciza squamosa*, henceforth referred to as SA) and the White-bibbed Antbird

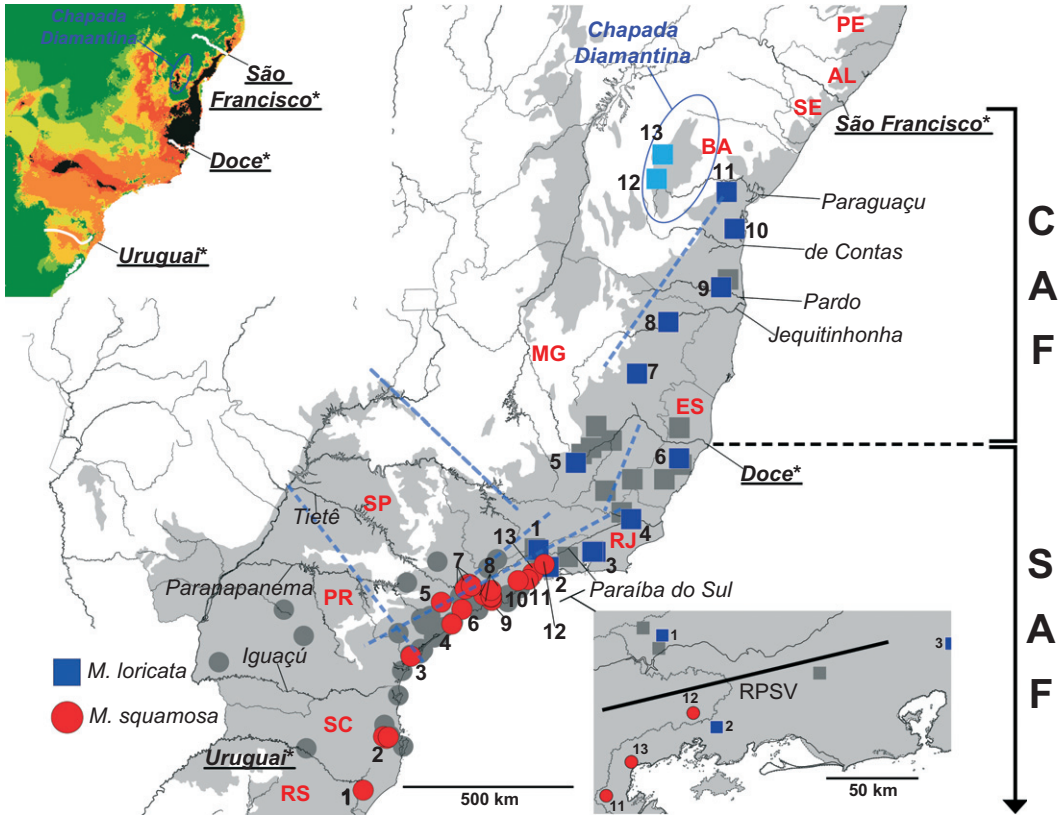


Fig. 1 Localities sampled in the present study (see also Fig. S2, Supporting information). Squares represent White-bibbed Antbird (WBA) and circles Squamate Antbird (SA). Coloured records represent genetic samples used, while historical (grey) show species distributions. The original, preclearing distribution of the Atlantic Forest (AF) is indicated in light grey, state borders in dark grey and major rivers in black. The main rivers in the area (Paraguaçu, de Contas, Pardo, Jequitinhonha, Doce, Paraíba do Sul, Tietê, Paranapanema, and Iguaçú) are indicated. The upper inset shows the forest modelled after a recent stability-extinction model for the AF (Fig. 3b from Carnaval & Moritz 2008; AF broader definition—see original reference for additional details and also a more specific definition of the AF), indicating both Central Atlantic Forest (CAF) and Southern Atlantic Forest (SAF). Areas in black and orange show regions of most probable predicted stability, while areas in yellow and green show regions of lesser and least probable predicted stability. Acronyms of states originally covered by the AF are indicated in red. Dashed lines indicate areas of putative geographic barriers contained in WBA/SA distribution, according to Thomé *et al.* (2010). The lower inset indicates the area of the mutual distribution limit of WBA and SA, as well as the River of Paraíba do Sul Valley. Light blue squares represent WBA localities identified in the STRUCTURE runs as the 'CD cluster' (see text).

(*Myrmeciza loricata*, WBA) are sister species (F. R. Amaral & C. Y. Miyaki, in preparation, G. Bravo & R. T. Brumfield, in preparation) of suboscine birds endemic to the Brazilian AF, where they inhabit both lowland and montane humid forests from sea level to 1000 m or higher (Ridgely & Tudor 2009). They occur in parapatry, and their combined distributions cover the central and southern portions of the AF, including most of the humid forest south of the São Francisco River (Fig. 1). Under the stability-extinction model, WBA's range includes both predicted areas of stability in the AF (the Bahia refugium, Fig. 1) as well as unstable areas south of the Doce River. However, SA's range lies entirely outside major refuges, in putatively unstable areas (Fig. 1). Thus, these two species are excellent models to test the effects of refuge isolation in the AF predicted by the stability-extinction model, as contrasting demographic histories would be expected for each species. In addition, many geologic faults and rivers not only dissect both species' distributions, but also roughly coincide with the area of their abutting ranges (Fig. 1), thus offering an opportunity to evaluate alternative processes of diversification (i.e. barrier hypotheses).

In addition to their utility as models for diversification processes in the AF, the WBA/SA species pair represents an interesting opportunity to study speciation of suboscine birds. Vocalizations are thought to be innate and very important in maintaining species integrity among antbirds (Isler *et al.* 1998), and for this reason, vocalizations have been used to help establish species limits—including in *Myrmeciza* antbirds (e.g. Chaves *et al.* 2010). However, WBA and SA are not only similar

in behaviour and plumage (males being especially alike, with females differing in throat and mask colour), but more remarkably, in vocalizations (Zimmer & Isler 2003). Their very similar songs associated with their parapatric distributions may raise questions concerning the strength of their reproductive isolation (see Fig. S1, Supporting information for sonograms).

Here we explore patterns of intra- and interspecific variation of WBA and SA using one of the largest sequence-based multilocus data sets used so far in a study of Neotropical phylogeography (nine putatively unlinked regions). We address the question whether patterns of divergence and historical demography fit the expectations of refuge isolation and/or the barrier hypotheses in the case of WBA and SA. In addition, due to their phenotypic similarities—vocalization in particular—and parapatry, we further ask whether multilocus data support recognition of WBA and SA as distinct evolutionary units.

Methods

Population and marker sampling

We obtained 89 antbird samples (48 WBA and 41 SA) from 26 localities (13 for each species), comprising one to nine individuals per locality (Fig. 1 and Table S1, Supporting information). Fieldwork in Brazilian National Parks and private land (2008–2010) provided most of the specimens, but additional samples were obtained from tissue collections. Birds were collected using the method described in Amaral *et al.* (2012a). Most individuals were represented by vouchered specimens (86 of 89). Species identification followed Ridgely & Tudor (2009).

We obtained sequences from nine markers, including eight anonymous nuclear loci designed specifically for SA (Amaral *et al.* 2012b, 268–422 bp), as well as complete sequences of the mitochondrial gene NADH subunit 2 (ND2, 1041 bp) using primers described elsewhere (for mitochondrial primer sequences and references see Table S2, Supporting information). Sequences were deposited in GenBank under the accession numbers KC714092–KC715586.

DNA extraction and sequencing

DNA was extracted using a modified version of the phenol–chloroform method of Bruford *et al.* (1992) as described by Tavares *et al.* (2006), or the DNeasy kit (Qiagen Inc.) according to the manufacturer's protocol. PCR was performed in 25 μL reactions, containing 2.5 μL of buffer 10× (Pharmacia), 1 μL of dNTP mix (2 μM each), 0.5 U of Taq-polymerase (Pharmacia), 1 μL of each primer (10 μM, Table 1) and 25–50 ng of DNA. Thermal cycling conditions for all markers were an initial denaturation step of 95 °C for 5 min, followed by 40 cycles of 95 °C (30 s), 60 °C (30 s) and 72 °C (40 s), and a final extension step of 72 °C of 10 min. Single-band products were purified using polyethylene glycol (PEG) precipitation or EXO-SAP enzymatic purification and sequenced using Big Dye terminator 3.1 cycle sequencing kit (Life Technologies), according to the manufacturer's protocol. The primers used for initial PCR and occasional allele-specific PCR (AS-PCR) were used for sequencing (Tables S2 and S3, Supporting information). Sequences were obtained using an ABI 3100, 3130 or 3730 automated sequencer (Life Technologies).

Both strands were assembled in contigs, and sequences were inspected and corrected by eye using Codoncode Aligner 3.7.1 (Codoncode Inc.). Heterozygous positions were first coded according to the IUPAC code. Individuals exhibiting alleles with indels had their heterozygous positions resolved with the method of Flot *et al.* (2006) as implemented in Champuru (Flot 2007) or using AS-PCR. Sequences were aligned in Clustal X 2.1 (Larkin *et al.* 2007) using default parameters and had their edges trimmed to equal sequence lengths in Bioedit 7.0.8 (Hall 1999).

Haplotype estimation, recombination, descriptive statistics and neutrality tests

Phased haplotypes were initially estimated computationally using Bayesian methods as implemented in PHASE (Stephens & Donnelly 2003). Gametic phases inferred with posterior probabilities equal or higher than 0.6 were considered resolved (Harrigan *et al.* 2008), and gametic phases with lower posterior probabilities were resolved using AS-PCR.

We tested for signs of past recombination using a PHI Test (Bruen *et al.* 2006) as implemented in SplitsTree4 (Huson & Bryant 2006). In case of a significant signal of recombination, the marker was reduced to the largest nonrecombining block based on the four-gamete test (Hudson & Kaplan 1985) performed in DNAsp 5.1 (Librado & Rozas 2009).

Nucleotide diversity (π), haplotype diversity (Hd), number of haplotypes (h) and number of segregating sites (S) were calculated for each marker and species. We tested for statistically significant differences in diversity estimators between species for each estimator using Mann–Whitney two-tailed tests using R 2.13.1 (R Development Core Team 2013). To detect significant deviations from the null hypothesis of neutral evolution and constant population size, we performed Tajima' D tests (Tajima 1989), Fu's *Fs* (Fu 1997) and R^2 tests

Table 1 Descriptive statistics and neutrality tests for each marker separated by species and STRUCTURE cluster. Bold numbers with asterisks indicate statistically significant results based on coalescent simulations. WBA non-CD does not include locality 9 (see STRUCTURE results for explanation)

	N	Length (bp)	π	Hd	S	h	Tajima′ D	Fu's *Fs*	Ramos & Rozas's R^2
ND2									
WBA	47	1041	0.00317	0.946	26	21	−1.455	**−10.829***	**0.058***
WBA/CD cluster	6	1041	0.00211	0.867	6	4	−0.932	−0.326	0.248
WBA/non-CD cluster	36	1041	0.00302	0.927	21	16	−1.282	**−6.467***	0.070
SA	40	1041	0.00072	0.571	7	9	−1.509	**−6.181***	**0.057***
Mysq-AL2									
WBA	96	72	0.01387	0.687	6	8	−0.328	−2.111	0.083
WBA/CD cluster	12	72	0.01178	0.667	2	3	0.822	0.360	0.212
WBA/non-CD cluster	74	72	0.01409	0.673	6	8	−0.415	−2.373	0.085
SA	82	72	0.00161	0.116	1	2	−0.505	−0.271	0.058
Mysq-AL8									
WBA	94	382	0.00151	0.490	7	8	−1.369	**−4.523***	0.043
WBA/CD cluster	12	382	0.00206	0.667	2	3	0.554	0.217	0.197
WBA/non-CD cluster	74	382	0.00127	0.416	6	7	−1.435	**−4.284***	0.047
SA	82	368–382	0.00362	0.701	8	8	−0.430	−1.213	0.082
Mysq-AL16									
WBA	96	68	0.00092	0.041	3	3	−1.614	−3.211	0.075
WBA/CD cluster	12	68	0.00000	0	0	1	—	—	—
WBA/non-CD cluster	74	68	0.00119	0.054	3	3	−1.650	−2.836	0.086
SA	82	68	0.00202	0.137	1	2	−0.378	−0.049	0.069
Mysq-AL17									
WBA	94	412	0.00440	0.744	12	13	−0.607	−3.619	0.074
WBA/CD cluster	12	412	0.00338	0.439	4	3	0.184	1.403	0.168
WBA/non-CD cluster	72	412	0.00374	0.691	12	13	−1.054	**−5.370***	0.063
SA	82	412	0.00141	0.529	5	6	−0.932	−2.258	0.065
Mysq-AL18									
WBA	96	350	0.00234	0.604	11	11	−1.622	**−6.527***	**0.038***
WBA/CD cluster	12	350	0.00429	0.742	4	5	0.466	−0.903	0.180
WBA/non-CD cluster	74	350	0.00209	0.575	8	8	−1.409	**−3.752***	0.046
SA	82	351	0.00060	0.205	3	4	−1.208	**−2.543***	0.048
Mysq-AL22									
WBA	92	267–268	0.00587	0.768	8	8	−0.014	−0.495	0.097
WBA/CD cluster	12	268	0.00384	0.667	2	3	1.624	0.747	0.258
WBA/non-CD cluster	70	267–268	0.00631	0.777	7	7	0.385	0.167	0.119
SA	80	267–268	0.00393	0.518	4	5	0.621	0.447	0.131
Mysq-AL23									
WBA	92	302	0.00483	0.822	10	12	−0.667	**−4.061***	0.072
WBA/CD cluster	12	302	0.00492	0.788	5	5	−0.380	−0.927	0.137
WBA/non-CD cluster	70	302	0.00468	0.815	9	11	−0.643	**−3.837***	0.078
SA	82	299–302	0.00000	0.000	0	1	—	—	—
Mysq-AL25									
WBA	94	309–311	0.01610	0.942	24	28	0.045	**−8.502***	0.101
WBA/CD cluster	12	311	0.01540	0.879	13	7	0.475	−0.130	0.171
WBA/non-CD cluster	72	309–311	0.01592	0.937	21	24	0.246	**−6.605***	0.114
SA	82	311	0.00328	0.590	10	9	−1.296	−3.183	0.058
Mean polymorphism									
WBA			0.00589	0.672	12	12			
WBA/CD cluster			0.00531	0.635	4	4			
WBA/non-CD cluster			0.00581	0.652	10	11			
SA			0.00191	0.374	4	5			
Mean nuclear polymorphism									
WBA			0.00623	0.637	10	11			
WBA/CD cluster			0.00571	0.606	4	4			

Table 1 *Continued*

	N	Length (bp)	π	Hd	S	h	Tajima' D	Fu's *Fs*	Ramos & Rozas's R^2
WBA/non-CD cluster			0.00616	0.617	9	10			
SA			0.00206	0.350	4	5			

CD, Chapada Diamantina; h, number of haplotypes; Hd, haplotype diversity; π, nucleotide diversity; S, number of segregating sites; SA, Squamate Antbird; WBA, White-bibbed Antbird.

(Ramos-Onsins & Rozas 2002). Significance was obtained based on 1000 coalescent simulations. All tests and simulations were conducted with DNAsp 5.1.

Population structure

Median-joining networks were inferred using the software NETWORK (Fluxus-Engineering) to explore general patterns of variation for each marker. Population structure was assessed with STRUCTURE 2.3.1 (Pritchard *et al.* 2000) using nuclear markers only, with alleles used instead of individual SNPs. Three data sets were examined: one with all samples from both species, and each species separately. We adopted the admixture model, using both models of independent and correlated allele frequencies. Twenty independent runs were performed for 10 values of *K*, using 500 000 generations burn-in and 5 000 000 total generations. To detect subtle substructure, the single species runs were also analysed using the LOCPRIOR model (Hubisz *et al.* 2009) and locality information (Table S1, Supporting information). A posteriori choice of the best *K* was performed with the ad hoc method proposed by Pritchard *et al.* (2000). Each set of runs per *K* was averaged using CLUMPP (Jakobsson & Rosenberg 2007). In order to evaluate concordance between the population structure found in STRUCTURE and mtDNA variation, we performed analyses of molecular variance—AMOVA (Excoffier *et al.* 1992), as implemented in Arlequin 3.5 (Excoffier *et al.* 2005), with the groupings found in STRUCTURE assumed as populations. Significance was estimated based on 50 000 permutations. Localities represented by only one individual were excluded from AMOVA tests.

Inferences of demographic history

We explored the speciation history of both species by fitting the data set to a model of isolation with migration as implemented in IMa2 (Nielsen & Wakeley 2001; Hey & Nielsen 2007; Hey 2010). The HKY (Hasegawa *et al.* 1985) model was applied for all markers. Several runs were performed in order to establish the best priors for effective population size, time of divergence and migration parameters. The final run was performed using 2 000 000 generations of burn-in, 100 000 trees sampled during 1 000 000 generations, and use of 20 chains (command line: -q10.0 -t8.0 -m1.0 -b2000000 -l100000 -d10 -p234567 -hfg -hn20 -ha0.96 -hb0.9). The estimated parameters were converted to demographic units assuming substitution rate ranges for ND2 from 0.8×10^{-8} substitutions/site/year (Fleischer *et al.* 1998), and for anonymous loci from the mean of 0.135×10^{-8} substitutions/site/year (Ellegren 2007). Conversions were also performed using several additional substitution rates, in order to evaluate the impact of rate choice on estimates (Table S4, Supporting information). We assumed a generation time of 1 year. Sensitivity of results to marker sampling was evaluated by performing runs using the total data set, as well as mtDNA-only and nDNA-only data sets.

Increasing effective population size through time is one of the strongest expectations of the refuge model, but not of the barrier hypothesis. We performed GMRF Skyride Plots (GSP, Minin *et al.* 2008) for mtDNA-only data sets and Extended Bayesian Skyline Plots (EBSP) for the nuclear multilocus data (Heled & Drummond 2008), which provide historical estimates of population size fluctuations. We fitted a HKY model for all markers and performed runs based on groups found in STRUCTURE. Invariant markers were excluded. We adopted a strict molecular clock and uniform priors on substitution rates ranging from 0.8 to 1×10^{-8} substitutions/site/year for ND2 (Fleischer *et al.* 1998; Lovette 2004) and $0.12–0.256 \times 10^{-8}$ substitutions/site/year for nuclear markers (Ellegren 2007; Lee & Edwards 2008). Chains were run for 500 million generations, with sampling performed at each of 25 000 steps.

Approximate Bayesian Computation

Because some results from the IMa2 analyses were equivocal (i.e. we were not able to estimate divergence times in WBA-only runs, and very small migration rates were inferred between WBA and SA), we also used Approximate Bayesian Computation (ABC) as a method for demographic inference to estimate the age of divergence within WBA and to test potential gene flow

between SA and WBA (for review, see Beaumont *et al.* 2002; Beaumont 2010; Csilléry *et al.* 2010; Tsai & Carstens 2013). We focused on nuclear variation in the ABC analyses to avoid analytical difficulties related to the divergent rates of evolution of mitochondrial and nuclear sequences. Two demographic scenarios were considered: a reproductive isolation model and a post-divergence migration model, both representing potential outcomes of vicariance followed by secondary contact. In both models, the times of divergence within the WBA complex were estimated under a uniform prior distribution (0–2 million years ago). Scenarios only differed in an additional parameter in the migration model, included as postdivergence, bidirectional migration between SA and the adjacent WBA cluster under a uniform prior (0–1 diploid individuals per generation), occurring after divergence within the WBA complex. Between-species gene flow was excluded in the isolation model. Remaining demographic parameters were set to values inferred using IMa2 for nuclear data. Four diversity estimators (nucleotide diversity, haplotype diversity, number of haplotypes and number of segregating sites) served as summary statistics for the ABC analysis. These measures were obtained for SA and each WBA cluster as well as the combined population total. In addition, we calculated pairwise F_{ST} (Hudson *et al.* 2002) between each pair of SA and WBA clusters. For each summary statistic, we used the mean of single-marker measures obtained for the nuclear data set. Both speciation scenarios were simulated under the coalescent process using the programme Bayesian Serial SimCoal (BayeSSC; Anderson *et al.* 2005), which is a modification of SimCoal 1.0 (Excoffier *et al.* 2000), and the analysis was performed using the abc package in R (Csilléry *et al.* 2012).

Before estimating demographic parameters, we used ABC for model selection, using half a million simulations per model. Posterior probabilities for each model were obtained using the logistic regression method (Beaumont 2008) as well as the simpler rejection-sampling method (Tavaré *et al.* 1997), whereas support for each model was compared by calculating Bayes factors from obtained probabilities (Kass & Raftery 1995; Robert 2007). The subsequent ABC parameter estimation step was carried out for the better supported demographic model (the isolation model), for which we obtained one million simulations. We used the weighted local-linear regression algorithm (Beaumont *et al.* 2002) and applied a correction for heteroscedasticity as implemented in the abc package. Because the regression adjustment can potentially result in posterior distributions that exceed the bounds of the corresponding prior distributions, it is sometimes recommended to transform parameters (Beaumont *et al.* 2002; Blum & François 2009). Therefore, following Hamilton *et al.* (2005), we applied a log-tangent transformation of parameters before regression. The resulting posterior distribution was back-transformed to record point estimates and the corresponding 95% highest posterior density (HPD) interval. Inferred demographic parameters were eventually confirmed by conducting back simulations.

Results

Genetic diversity

From a potential total of 801 sequences (89 individuals × 9 markers), all but six were successfully obtained and, if nuclear, had gametic phases resolved. Statistically significant PHI tests indicated signs of recombination in two markers, Mysq-AL2 and Mysq-AL16, which were reduced to 72 and 68 bp from alignments of 346 and 422 bp, respectively.

Descriptive statistics (Table 1) indicated that ND2 variation was higher than the mean nuclear polymorphism in most measures, except for nucleotide diversity. Variation of two nuclear markers (SA: Mysq-AL8 and Mysq-AL 25) was also higher than that of ND2 in number of segregating sites and haplotype diversity. No marker showed significant deviation from the null hypothesis of neutrality and constant population size according to Tajima's D test. Significant values of R^2 and/or Fu's *F*s tests, however, were obtained in some cases (ND2: WBA and SA; Mysq-AL8: WBA; Mysq-AL17: WBA; Mysq-AL18: WBA and SA; Mysq-AL23: WBA; and Mysq-AL25: WBA; see Table. 1). Levels of genetic diversity were significantly higher in WBA than in SA in all variables measured, namely nucleotide diversity ($W = 64$, $P = 0.04$), haplotype diversity ($W = 66$, $P = 0.02$), number of haplotypes ($W = 67.5$, $P = 0.02$) and number of segregating sites ($W = 68$, $P = 0.02$).

Population structure

Median-joining networks suggest very limited shared variation between WBA and SA (Fig. 2). In five markers, the species share no variation (ND2, Mysq-AL8, Mysq-AL18, Mysq-AL22 and Mysq-AL23), while in the remaining markers (Mysq-AL2, Mysq-AL16, Mysq-AL17 and Mysq-AL25), they share a maximum of two alleles. ND2 haplotypes of WBA and SA are separated by 46 substitutions (or a Kimura 2-Parameter net divergence of 4.8%). A minimum of one to four substitutions separate WBA from SA alleles of nuclear markers lacking shared variation. The reciprocal monophyly of most of the markers (6 of 9) underscores the substantial divergence of this species pair.

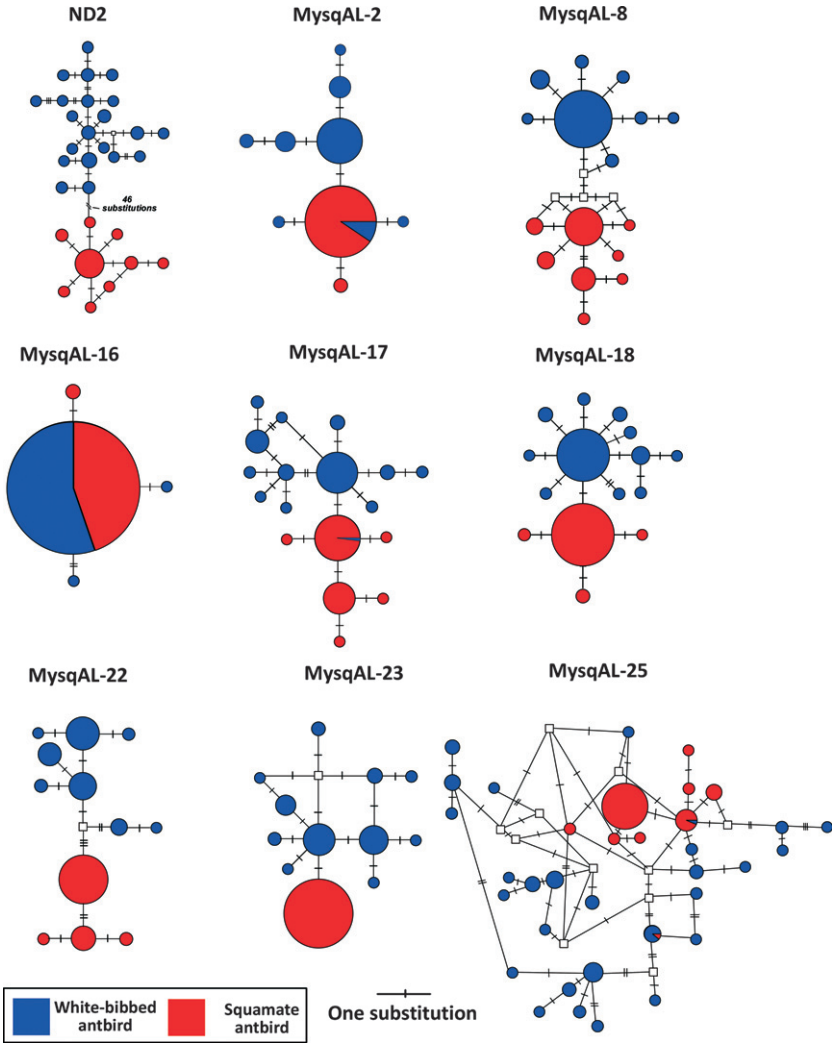


Fig. 2 Median-joining networks inferred for data sets of combined White-bibbed Antbird and Squamate Antbird samples. Squares represent inferred haplotypes/alleles.

STRUCTURE analyses based on all individuals recovered two clusters that match allocation of individuals to WBA and SA, irrespective of number of K or model used (Fig. 3 and Fig. S2, Supporting information). Admixture appears to be very limited or nonexistent, and no individual presented ancestry coefficients of less than 96.2% to its respective cluster when $K = 2$ is considered, independently of the model adopted. Based on the ad hoc method of Pritchard *et al.* (2000), $K = 2$ is the best choice under the model of admixture with independent allele frequencies, while $K = 5$ is favoured under the model of admixture with correlated allele frequencies. When species were analysed separately, all runs had $K = 1$ as best choice for SA. The best number of clusters for WBA varied among from $K = 1$ (admixture with correlated allele frequencies), $K = 2$ (LOCPRIOR + admixture with independent or correlated allele frequencies) and $K = 3$ (admixture with independent allele frequencies). Runs for WBA based on LOCPRIOR and independent allele frequencies identified a cluster composed of individuals 43–48, corresponding to the region of Chapada Diamantina (henceforth referred to as CD), that is, localities 12 and 13 (Figs 1 and 3 and Fig. S2, Supporting information). The same clustering appeared when LOCPRIOR with a model of correlated allele frequencies was used, but in this case, the cluster also included individuals 34–38 (from locality 9). Given the discordance across models, which could result from introgression between CD and non-CD clusters or simply lack of statistical power to establish the boundaries between those two clusters, we focused on the cluster of individuals that was present in most STRUCTURE runs, namely the CD cluster (individuals 43–48; Fig. 3 and Fig. S2, Supporting information) and excluded WBA locality 9 from most downstream analyses (except in ABC). AMOVA combining SA and WBA indicate that 95.3% of mtDNA variation is explained by interspecific differences ($P < 0.01$). Intraspecific AMOVA performed between WBA's CD and non-CD clusters (excluding locality 9) indicates that

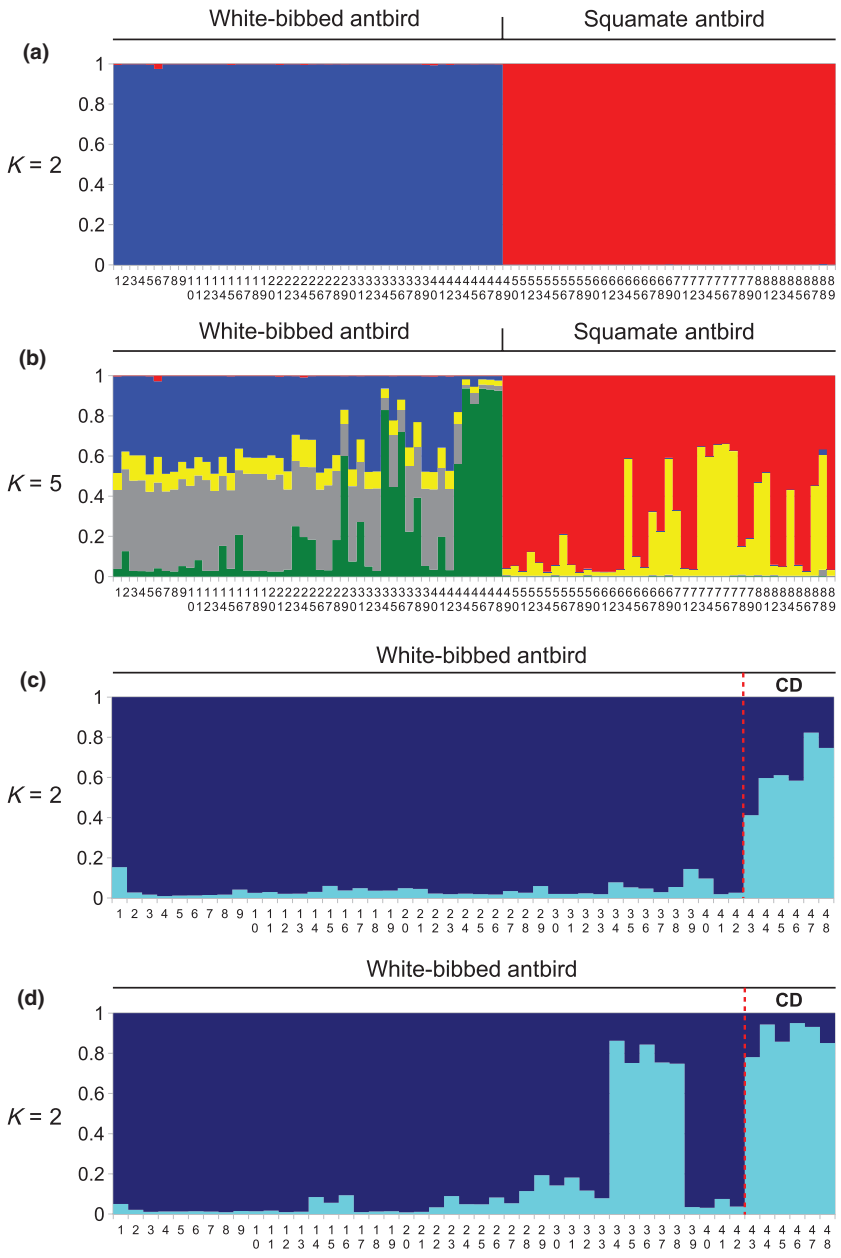


Fig. 3 STRUCTURE plots depicting population structure between samples of White-bibbed Antbird (WBA) and Squamate Antbird (a and b), and substructure within WBA (c and d). Plots shown represent the best K according to the ad hoc method after Pritchard *et al.* (2000). Panels (a) and (b) result from runs under the models of admixture with independent allele frequencies and admixture with correlated allele frequencies, respectively. Panels (c) and (d) result from runs under the models of LOCPRIOR plus admixture with independent allele frequencies and LOCPRIOR plus admixture with correlated allele frequencies, respectively. The red dashed line in (c) and (d) marks WBA's Chapada Diamantina cluster. See Fig. S2 (Supporting information) for additional plots.

24.0% of mtDNA variation is due to among-cluster differences ($P > 0.05$).

Divergence population genetics

Isolation-with-migration (IMa2) analyses using the total data set (Fig. 4 and Table S4, Supporting information) suggest that divergence between species occurred at 3.8 million years ago (mya) and a 95% Highest Posterior Density (HPD) interval ranging from 2.2 to 5.4 mya. Estimates of effective population size of WBA (1.9×10^6 individuals, 95% HPD: 1.4×10^6–2.4×10^6) are more than four times larger than for SA (0.4×10^6 individuals, 95% HPD: 0.3×10^6–0.6×10^6). The estimated 95% HPD interval for ancestral effective population size is large (8400–1.3×10^6 individuals) and encompasses the 95% HPD interval of SA, but is lower and does not overlap with the 95% HPD interval estimated for WBA. Population migration rates are nonzero in both directions, being almost 10 times larger from SA to WBA (*2NM* maximum-likelihood = 0.104, 95% HPD: 0.026–0.261) than from WBA to SA (*2NM* maximum-likelihood = 0.017, 95% HPD: 0.001–0.079). Both rates are significantly different from zero according to the likelihood ratio test of Nielsen & Wakeley (2001), implemented in IMa2. In comparison with runs based on the

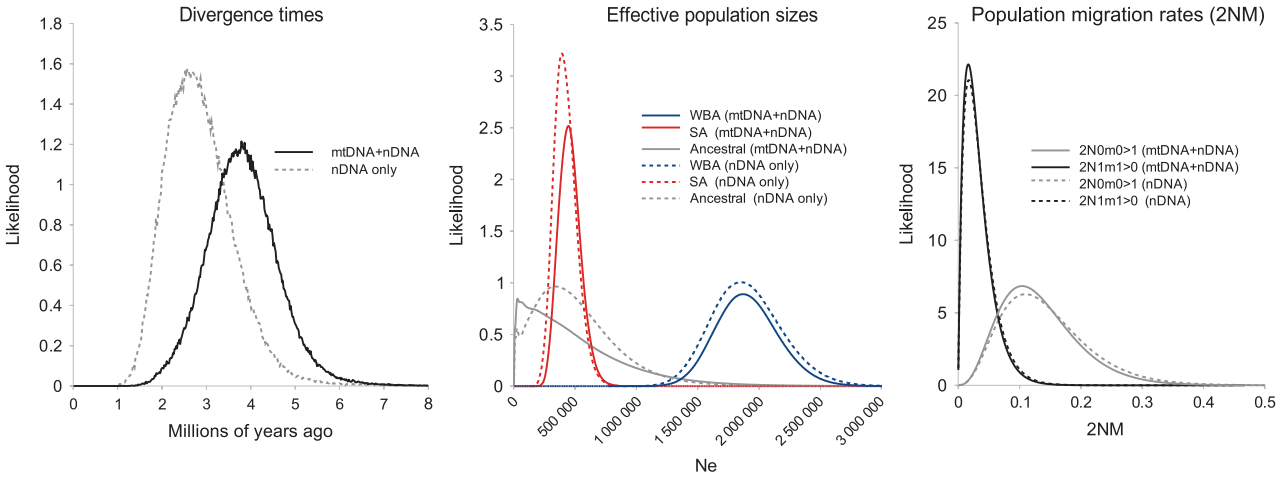


Fig. 4 Isolation-with-migration plots. Solid lines represent runs based on the total data set, while dashed lines correspond to nuclear-only runs. Assumed rates were 0.008 substitutions/site/lineage/million years for mtDNA and 0.00135 substitutions/site/lineage/million years for nDNA, but see Table S4 (Supporting information) for alternative conversions.

total data set, IMa2 analyses based on nuclear markers only resulted in very similar point estimates and 95% HPD ranges of effective population sizes and Population migration rates (Fig. 4 and Table S4, Supporting information). However, divergence times were approximately 1 million years younger in the nDNA-only data set compared to results from the total data set (2.6 mya, 95% HPD: 1.5–4.4 mya). Runs based on mtDNA alone did not contain sufficient information to estimate parameters in the full model and were not considered.

Historical fluctuations of population size

Three clusters were analysed in separate runs to infer Bayesian Skyline and GMRF Skyride Plots: SA, WBA/CD and WBA/non-CD (Fig. 5). Given the requirement for panmixis to perform those inferences, individuals of WBA from locality 9 were not considered because their uncertain placement in the STRUCTURE runs could result from gene flow between CD and non-CD populations. Invariant markers were excluded for SA EBSP runs (Mysq-C23) and CD cluster EBSP runs (Mysq-C16). Based on medians of runs using the total data set (Fig. 5), there were no signs of historical population fluctuations in the WBA/CD cluster, while population size increase was inferred for both WBA/non-CD and SA. A gradual increase in the non-CD WBA may have begun at approximately 2 million years ago, while a sharp increase in population size for SA was inferred to have begun approximately 20 000 years ago. Nuclear-only EBSPs were similar to those obtained using the total data set. The mtDNA-only GSPs for the CD cluster and SA were similar to those obtained with multilocus data, but with much shorter observation windows. The mtDNA-only estimates for non-CD WBA contrast with the long-term stability inferred using the total and nDNA-only data sets, although 95% HPD intervals are large and could accommodate a scenario of stability (i.e. a flat line). All mtDNA-only estimates presented an observation window of less than 500 000 years, compared to millions of years in the nDNA and mtDNA + nDNA estimates.

Intraspecific divergence and interspecific gene flow

Based on the clustering inferred using STRUCTURE, we included CD and non-CD WBA as demographic units besides SA in our ABC analysis. Here, we included WBA non-CD locality 9, because it could provide insights into gene flow estimates. With the estimates of diversity for each marker, the observed vector of summary statistics contained 19 mean values of single-locus statistics (four measures for each of the three groups and the population total, and one between each group). We evaluated two scenarios that differed in the level of cross-species gene flow as demographic parameter, that is, presence or absence of migration in a reproductive isolation model and postdivergence migration model, respectively. In the latter, bidirectional migration was included between SA and WBA/non-CD as adjacent cluster, after it diverged from the CD cluster. Under the logistic regression method for model selection in ABC, Bayes factors (BF) substantially supported the reproductive isolation model (BF = 4.92) over the postdivergence migration model (BF = 0.20, see Fig. 6a). This result was further emphasized when the analysis was carried out using the rejection-sampling method (BF = 8.86 and 0.11, for isolation and migration, respectively). Under the isolation model, the time of divergence of CD and non-CD WBA was esti-

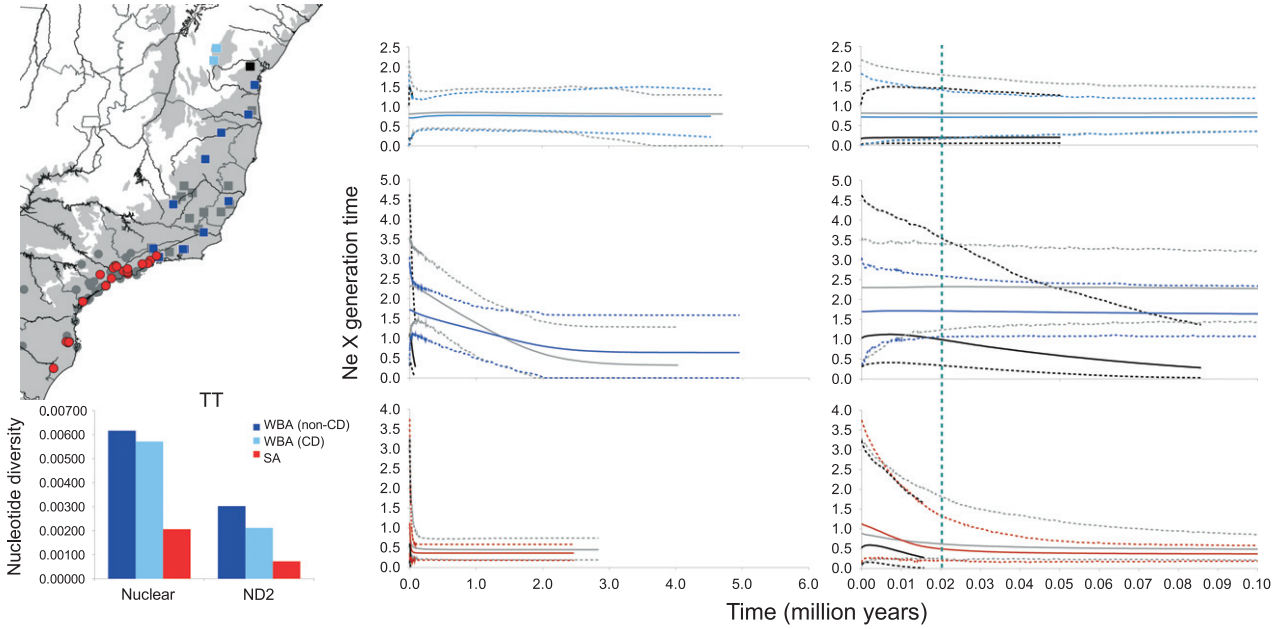


Fig. 5 Extended Bayesian Skyline and GMRF Skyride Plots. Color lines represent EBSPs based on mtDNA + nDNA data, light grey lines represent nuclear-only EBSPs and black lines represent mtDNA GSPs. The same runs are presented side by side using different scales for a detailed view of the last 100 000 years. The map colours represent White-bibbed Antbird (WBA)/Chapada Diamantina (CD) cluster (light blue), WBA/non-CD cluster (dark blue) and Squamate Antbird (red). WBA's locality 9, which has been excluded from those analyses (see text for reasons), is represented in black. Nucleotide diversity is also illustrated.

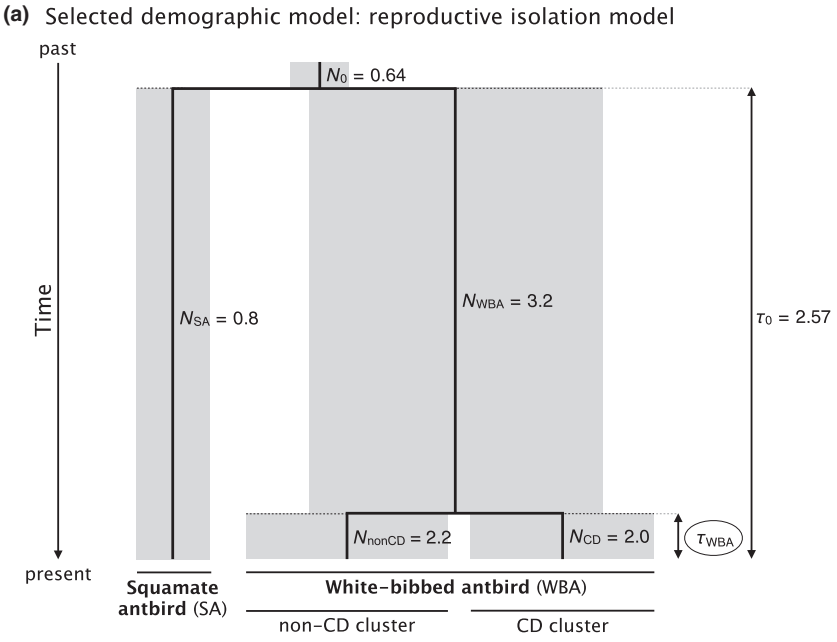
mated at around 135 000 YBP (95% HPD: 42 000–1 388 000) when taking the mode as point estimate, or even earlier when considering the median (249 000 YBP) or mean (359 000 YBP) of the posterior distribution (Fig. 6b). As the migration model was not supported, no gene flow between SA and the adjacent WBA/non-CD cluster was found to be present after the divergence of the CD/non-CD divergence.

After parameter estimation, we assessed these results in a model checking procedure, whereas the inferred divergence time was fixed in the isolation model, and replicate simulations were performed under the coalescent process to obtain posterior predictive distributions for each summary statistic. Observations falling outside or in the far end of tails (e.g., $P \leq 0.025$) of the predictive distributions suggest that parameters could not be appropriately estimated, most likely due to an insufficient choice of summary statistics and/or an inappropriate demographic model (Beaumont 2010). However, none of the observed summary statistics appeared outside of its posterior predictive distribution (Fig. S3, Supporting information), and the probabilities of observations to falling in either tail of the distribution were all $P \geq 0.1$ (mean P-value: 0.32). Thus, our results indicate that both the demographic model used and the divergence time inferred were adequate to approximate the underlying evolutionary history of the WBA/SA species pair.

Discussion

Myrmeciza *antbirds: evidence of refugia, barriers or both?*

In our study of two species of endemic antbirds, we tested alternative scenarios of diversification in the hyper-diverse Atlantic Forest (AF) biome based on the largest sequence-based data set of an AF endemic organism thus far. Our results fulfil expectations of the refuge theory as elaborated in the stability-extinction scenario for the recent history of the AF biota as recently proposed by Carnaval & Moritz (2008) and Carnaval *et al.* (2009). In support of this model, we found: (i) strong demographic fluctuations coinciding with the LGM in the Squamate Antbird (SA), which occurs entirely in portion of the AF with least forest stability (Southern AF); (ii) long-term population growth not coinciding with LGM or stability (CD cluster and non-CD cluster, respectively) in the White-bibbed Antbird (WBA), which has most of its distribution in a large putative refuge (Bahia); (iii) higher genetic diversity in WBA compared to the SA, reflecting expected differences in persistence in species occupying northern and southern portions of the AF; (iv) persistence in areas of predicted refuges, as indicated by population structure in WBA; and (v) evidence of secondary contact, based on parapatric



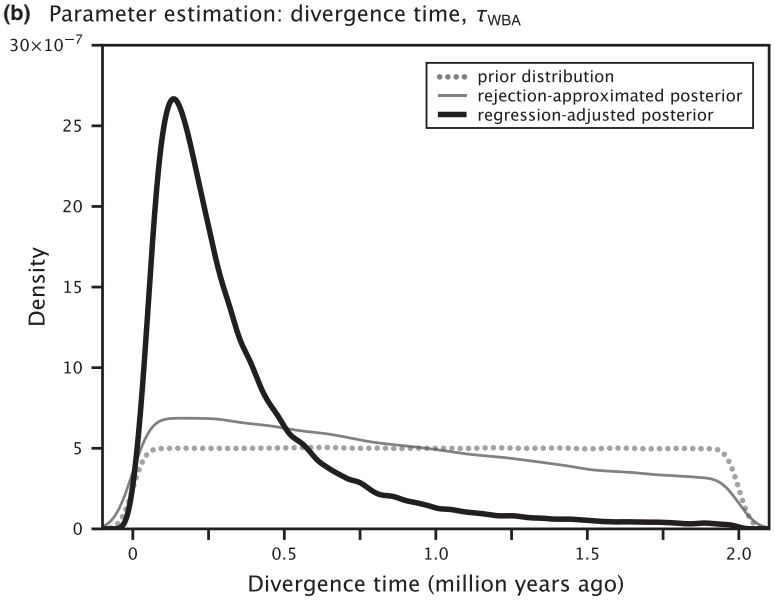


Fig. 6 Results of the model selection and parameter estimation procedures in Approximate Bayesian Computation (ABC). (a) Schematic representation of the reproductive isolation model, which had more explanatory power than the postdivergence migration model, as revealed by model selection in ABC. The model is represented as phylogenetic tree (thick black lines), whereas each branch is labelled with the effective population size (N_{pop}), in units of a million diploid individuals, and the width of each branch (shaded area) is proportional to the indicated size. Adjacent vertical lines illustrate the time to divergence (τ_{pop}) with labels in units of a million years ago, whereas the splitting time between Chapada Diamantina (CD) and non-CD White-bibbed Antbird (WBA) (circled) was included as a prior distribution in the model, and its value was estimated in the subsequent parameter estimation step. (b) Posterior distribution found when estimating the divergence time of CD and non-CD WBA under the reproductive isolation model, with rejection sampling of the prior distribution (thin grey line) and the posterior distribution achieved by local-linear regression (thick black line). The prior distribution itself is indicated (dashed line) as reference.

distributions of WBA and SA associated with signs of population expansion. One aspect of our data, however, does not completely fit the stability-extinction scenario. According to that model, species currently inhabiting the Southern AF should represent recent offshoots from expansion of northern populations. Instead, our data suggest that SA is a divergent evolutionary unit endemic to the Southern AFs, echoing similar findings with other organisms (e.g. Grazziotin *et al.* 2006; Cabanne *et al.* 2007; Fitzpatrick *et al.* 2009; Thomé *et al.* 2010; D'Horta *et al.* 2011; Amaro *et al.* 2012; Maldonado-Coelho 2012). Because avian distributions are labile and may change at timescales as short as a human lifespan (Brumfield 2012), we cannot rule out a scenario in which SA survived by shifting its distribution to northern latitudes during glacial periods. However, if major range shifts did not occur, it is possible that SA may have been persisting in Southern AF for millions of years since their initial divergence from WBA. Conceivably the original stability-extinction model lacks predictive power for the southern portions of the biome (Amaro *et al.* 2012), as exemplified by inference of many taxon-specific refuges in southeastern and Southern Brazil (Porto *et al.* 2013). Additionally, persistence in Southern

AF refuges has been hypothesized to be strongest in species capable of surviving in montane forest (as WBA and SA), because their tolerance to colder climates could shield them from the effects of the LGM (Amaro *et al.* 2012). Thus, although not originally predicted by the original stability-extinction model, recent evidence suggests that lineage persistence in the Southern AF—as may have happened with the SA—may also be consistent with a scenario of refuge isolation.

Our results also suggest that a small region west of the large Bahia refuge, the Chapada Diamantina uplands, harbours a population of WBA with signs of differentiation from the rest of the species' range. This area contains a small patch of AF harbouring populations of otherwise typically AF-inhabiting species as well as endemic organisms (e.g. Gonzaga *et al.* 2007), is mostly surrounded by open *Caatinga* vegetation, and still remains little explored in phylogeographic studies. A role for the Chapada Diamantina uplands as a small putative refuge in the stability-extinction model (Fig. 1) is consistent with the inferred stability and differentiation of the Chapada Diamantina (CD) cluster found in the present study. However, the localities sampled here are also separated from the rest of WBA's range by a potential riverine barrier, the Paraguaçu River (Fig. 1). Thus, the hypothesis of this river acting as a barrier cannot be rejected here, and further sampling in the CD area south of the Paraguaçu River will be necessary to discriminate between these hypotheses. Additional sampling of individuals in CD and neighbouring areas would also improve estimates of population structure, which in turn would provide more precise boundaries between CD and non-CD cluster (e.g. resolving the status of individuals of WBA locality 9).

Besides the unresolved role of the Paraguaçu River, on recent timescales our results do not support riverine or physiographic barriers as drivers of population structure, despite the many other rivers and topographic features that currently dissect the ranges of WBA and SA (Fig. 1). However, barriers may have acted on older timescales, namely during the initial divergence between SA and WBA. The antbirds studied here appear to have diverged well before the Pleistocene (2–4 mya), and their shared distribution boundary matches an area of high tectonic activity during the Miocene and Pliocene namely the River of Paraíba do Sul Valley (Petri & Fúlfaro 1983; Fig. 1). Alternatively, refuge isolation may have been responsible for both initial divergence and evolution of intraspecific patterns of variation, because climatic fluctuations may have been important even prior to the Pleistocene (Haffer 1993; Hewitt 1999). Thus, although intraspecific patterns of variation of WBA and SA strongly support the effect of refuge isolation in the AF, we cannot yet reject a role of the barrier hypothesis entirely, particularly during the initial divergence of WBA/SA on a larger timescale.

Vicariance, dynamic history or stochastic error? Why Atlantic Forest phylogeography demands multilocus data?

Taking at face value recent studies inferring divergence times and demographic histories of AF organisms performed using mtDNA or small (2–4) numbers of independent markers, what would the multilocus estimates of WBA/SA divergence suggest about assembly wide historical processes? Their pre-Pleistocene divergence is among the oldest reported, especially when compared to other AF avian sister species or mtDNA phylogroups. It is, for example much older than most of the divergences inferred for codistributed mtDNA lineages of suboscine songbirds, which usually diverged less than 1 mya (Cabanne *et al.* 2007, 2008; D'Horta *et al.* 2011; Maldonado-Coelho 2012). Our estimates are rivalled so far only by divergences estimated for species of *Eleoscytalopus* tapaculos (Mata *et al.* 2009). Our timing is in line with divergences found among codistributed clades of other vertebrates, such as frogs (Fitzpatrick *et al.* 2009; Brunes *et al.* 2010; Amaro *et al.* 2012) and pitvipers (Grazziotin *et al.* 2006). Thus, it is possible that temporally distinct vicariant events may have shaped AF vertebrate diversity, sometimes leading to pulses of diversification that may have affected distantly related organisms in similar ways.

Besides the apparent periodicity of large-scale events, sister lineages resulting from temporally independent events appear to accumulate in selected areas of the AF. The River of Paraíba do Sul Valley is a clear example, by roughly demarcating the shared range limits of phylogroups/species pairs that appear to have diverged very recently (<1 mya, Cabanne *et al.* 2007; D'Horta *et al.* 2011) or even before the Pleistocene (e.g. WBA vs. SA). Consequently, these areas should be investigated not only for their roles as primary generators of diversity, but also as suture zones, because many of those lineages may have not have diverged, but expanded simultaneously (Moritz *et al.* 2009; Martins 2011). Finally, our results are consistent with a number of recent studies suggesting that refuge isolation may have been an important mechanism of diversification in the recent history of the AF (Grazziotin *et al.* 2006; Cabanne *et al.* 2007; Carnaval *et al.* 2009; Fitzpatrick *et al.* 2009; Martins *et al.* 2009; Brunes *et al.* 2010; D'Horta *et al.* 2011; Maldonado-Coelho 2012). However, our findings do not reject the idea that barriers may have played an important role in the AF diversification, in particular, before the Pleistocene

(Pellegrino *et al.* 2005; Brunes *et al.* 2010; Thomé *et al.* 2010; Amaro *et al.* 2012).

Biases of single and multilocus demographic estimates in AF studies

Here, we address the impact of limited marker sampling, including possibilities of bias in this study, on estimates of demographic parameters in AF studies. In particular, we ask: Do available molecular studies—mostly based on mtDNA alone or a few markers—already provide a general framework to understand processes of AF diversification?

Divergence times. Theory predicts that comparison of divergence times across different studies based on one or few markers may not be straightforward, because gene divergence may largely pre-date population divergence (Edwards & Beerli 2000). Our results, for example, match previous empirical inferences (Lee & Edwards 2008) suggesting that estimation of divergence times may be affected not only by the number of loci, but also by whether or not mtDNA is included. In addition, recent studies suggest that natural selection may reduce mutation rates with increasing effective population sizes in selected prokaryotes and unicellular eukaryotes (Sung *et al.* 2012). If this process also affects birds, comparisons between species/populations with considerably distinct effective population sizes—as in our case—may be subject to this additional source of error when constant substitution rates are assumed to estimate divergence times.

Demography. Another essential aspect to be considered in comparisons across AF studies is the key role that historical demography plays in the AF, and more generally, Neotropical phylogeography. The statistical power of methods we have used, such as Extended Bayesian Skyline Plots (EBSP; Heled & Drummond 2008), as well as their 'observation window' depends on the number of loci used (Heled & Drummond 2008). Our results are in line with those predictions: on the one hand, the longest observation windows (spanning millions of years) and the most narrow 95% HPD intervals in effective population size estimations were obtained in multilocus comparisons (Fig. 5), which were much more informative than the single-locus GMRF Skyride Plot, whose observation windows were limited to much less than 500 000 years. Further, our data suggests that the medians of the combined data sets do not necessarily reflect the patterns found in mtDNA-only GSP and nuclear EBSPs analysed alone (e.g. non-CD WBA, Fig. 5). A possible explanation is that combining fast and slow evolving markers may be necessary to capture the greatest diversity of events, because specific demographic events may be concealed or revealed depending on which markers capture them (Eytan & Hellberg 2010). However, combining mtDNA and nuclear DNA is challenging because of their strongly divergent substitution rates, which are not well accommodated by some software. Thus, accurate reconstructions of demographic oscillations predicted by the refuge hypothesis will likely depend on the use of multilocus data sets and may possibly improve by combining mtDNA and many nDNA markers.

Divergence of WBA and SA, and implications for the speciation process in antbirds

The WBA/SA species pair comprises an interesting exception to most other closely related antbirds studied to date. Vocalizations have been considered one of the most important factors determining reproductive isolation among thamnophilids (Isler *et al.* 1998). WBA and SA are very similar in voice, and their parapatric distributions present a sharp transition of phenotypes within a distance of less than 20 km (Fig. 1). Considering that vocal differences are important to maintain species boundaries in such a geographic setting, very similar vocalizations, strong response to each other's voice and plumage similarities could lead to the prediction of little genetic divergence and/or high levels of gene flow between those two species. However, here, we found the opposite pattern, namely deep genetic divergence associated with low or nonexistent levels of gene flow. Thus, our results suggest that conspicuous vocal differences may not be always necessary to maintain species boundaries among antbirds, and other kinds of cues may be especially important in selected cases. Subtle vocal differences, which have been shown to suffice both interspecific and individual recognition in *Hypocnemis* antbirds (Seddon & Tobias 2010), or even visual cues (e.g. ochraceus-throated female WBA vs white-throated female SA) or non-vocal behaviours could be acting as strong prezygotic barriers. Alternatively, genomic incompatibilities caused by millions of years of divergence may have led to the development of mechanisms of postzygotic isolation (Price 2007).

But how does the WBA/SA pair interact at its zone of contact and what maintains their parapatry? Under a scenario of complete reproductive isolation, very similar ecological niches could be leading to competitive exclusion, even after substantial genetic divergence (Rundell & Price 2009; Weir & Price 2011). Alternatively, if reproductive isolation is not yet complete, a very narrow and still unidentified hybrid zone could exist, and strong selective forces against hybrids could be preventing substantial introgression and sympatry (Barton &

Hewitt 1985). Field and laboratory experiments, bioacoustic studies and genome-wide molecular surveys with this species pair will provide exciting findings on the development of reproductive isolation among antbirds, and more generally, suboscine passerines.

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F.R.A., P.K.A., S.V.E. and C.Y.M. conceived and drafted the manuscript. F.R.A. performed field and laboratory work. F.R.A., P.K.A. and S.V.E. performed data analyses. F.R.A., S.V.E. and C.Y.M. contributed with reagents.

Data accessibility

DNA sequences: GenBank accessions KC714092-KC715586

IMa2 and STRUCTURE input files: Dryad doi:10.5061/dryad.86hf6

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Sonograms of loudsongs and calls of SA (a and b) and WBA (c and d).

Fig. S2 Complete set of STRUCTURE runs.

Fig. S3 Model checking after model selection and parameter estimation in Approximate Bayesian Computation (ABC).

Table S1 Samples used in the present study.

Table S2 Mitochondrial PCR primers used for amplification and sequencing.

Table S3 PCR primers used for allele-specific PCR (AS-PCR).

Table S4 Conversion of Isolation-with-Migration parameters (population size and divergence times) based on different substitution rates obtained from the literature.

Table S5 Observed summary statistics used for Approximate Bayesian Computation (ABC).

Appendix S1 Details of ABC analysis.